ejpmr, 2021,8(12), 222-229

SJIF Impact Factor 6.222

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

GENES AND PROTEINS INVOLVED IN FORMATION OF VITAL ORGANS DURING DEVELOPMENT OF ZEBRAFISH WHEN EXPOSED TO DELTAMETHRIN

Kuder Reshma Shabnam[1*], Dharmapuri Gangappa[2] and Gundala Harold Philip*

*Department of Zoology, Sri Krishnadevaraya University, Anantapuram 515003, Andhra Pradesh, India.
[1]Department of Zoology, Sri Krishnadevaraya University, Anantapuram 515003, Andhra Pradesh, India.
[2]Department of Animal Biology, School of life sciences, University of Hyderabad 500046, Telangana, India.

***Corresponding Author: Kuder Reshma Shabnam**

Department of Zoology, Sri Krishnadevaraya University, Anantapuram 515003, Andhra Pradesh, India.

Article Received on 26/09/2021 **Article Revised on 15/10/2021** **Article Accepted on 05/11/2021**

ABSTRACT

Characterization of a protein of interest during development is essential for functional studies. A general strategy for understanding the function of a particular protein involves the generation of null mutations, or treatment with drugs, that interfere with its activity. To demonstrate that the synthesis, stability, or activity of a protein has been affected, accurate and efficient detection of low amounts of protein is essential. This can be achieved by immuno histochemistry or by western blot. Here we describe a method for the detection of proteins from single de-yolked zebrafish embryos. This procedure includes a fixation step and the concomitant elimination of lipids from the yolk cell. We show that this approach allows the rapid analysis of proteins in embryos without having to manually remove the yolk. This method provides a convenient alternative for genotyping of mutant embryos as early as the 128 cell stage. In addition, in drug- or morpholino-treated embryos, the correlation between the penetrance of a phenotype and the concentration of a protein present may be established.

KEY WORDS: Deltamethrin (DM), Zebrafish, no tail (*ntl),* floating head *(flh),* syu *(syu)* and doc (*doc*).

INTRODUCTION

Synthetic pyrethroid insecticides have generated public concerns due to their increasing use and potential effects on aquatic ecosystems.[1] Pyrethroids have also been used extensively for mosquito control following outbreaks of the West Nile virus, and have become the pesticide of choice to combat bedbug infestations (Agency for Toxic Substances and Disease Registry (ATSDR).[2] Deltamethrin, a type-II pyrethroid compound is one of the most potent insecticides known and is widely used in veterinary products to control lice, flies, and ticks on cattle, sheep, and pigs, as well as in agricultural formulations to control numerous insect pests on fruits, vegetables, and field crops.[3] In fish farms they are used to control ectoparasites and biological vectors.[4], [5] Even though pyrethroids are often considered to be ‘‘safer’’ pesticides because of their low to moderate acute toxicity to non-target species, their increased use raises concerns of potential adverse effects, particularly in sensitive populations such as children. This concern is intensified by recent studies indicating that children are exposed to pyrethroids during development.[6] Pyrethroid metabolites have been found in the urine of pregnant women.[7],[8] Lu et al.[9],[10] have o found pyrethroid metabolites in urine of elementary-age children that appear to be primarily the result of residential exposure. Morgan et al. [11] 2007 also found that 67% of cohort preschool children with detectable levels of the pyrethroid metabolite 3-Phenoxy Benzoic acid in their urine. Various medical abnormalities in Chinese population due to deltamethrin intoxication were also reported by (He et al.)[12]

Studies from our laboratory with DM have shown malformations like yolk sac and pericardial edema, dorsal curvature of the spine and decreased pigmentation in zebrafish embryos.[13] Another study by Shamshad begum et al.[14] has shown alterations of different proteins in five different tissues namely liver, brain, kidney, ovary and testis in adult zebrafish after exposure to 5µg/L of DM for 6days. Induction of vitellogenin in male fish and decrease of 17β-estradiol and 11-ketotestosterone in female and male fish respectively along with histological changes in both testes and ovary were demonstrated after treatment with DM.[15] We have also shown its effect on the activities of Catalase, Superoxide dismutase, Glutathione-S-transferase, Glutathione peroxidase and Lipid peroxidation in liver and ovary tissues of Zebrafish (*Danio rerio*).[16]

In present study, we sought to investigate the developmental toxicity of type II compound deltamethrin by determining the expression of four genes, *no tail* (*ntl), floating head (flh), Syu (Syu)* and *doc (doc)* involved in the formation of notochord trunk, somite containing myoseptum and floor plate development after exposing

4hpf embryos to two concentrations of DM till 48hpf stage.

The zebrafish (*Danio rerio*), has been choosen in the study as it is widely used as a prominent vertebrate model organism in different fields because of its small size, low cost, diversified adaptability, short breeding cycle, high fecundity and transparency of the chorionic membrane which allows stepwise developmental visualization.[17]

MATERIALS AND METHODS

Toxicant tested-preparation of stock solution

Technical-grade Deltamethrin [(S) α-cyano-3-phenoxybenzyl-(1R)-cis-3-(2,2 dibromovinyl)-2,2 dimethylcyclopropane-carboxylate)] which is of 99.7% purity (CAS NO. 52918-63-5) was purchased from Sigma-Aldrich. Stock solution was prepared by dissolving 10mg DM in 2mL ethanol (99.9%) and stored at 4°C in darkness. Daily requirement was taken from this. Concentrations were selected following initial-finding experiments at 50, 100, 200, 300 and 400µg/L. Significant survival rates were noticed only at 100 and 200µg/L concentrations.

Maintenance of adult zebrafish and embryo collection

Wild type adult Zebrafish (*Danio rerio*) used in this study were bred in our aquaria facility for two generations. Females and males in the age group of six to 12 months were kept in a ratio of 2:1 in an aquarium filled with filtered tap water with the oxygen saturation of more than 80% and pH at 7.0±0.3. The water temperature was maintained at 26±1^{0}C at a 14h:10h day and night regime. Fish were regularly provided with varied diet comprised of freshly hatched live brine shrimp (*Artemia nauplii*) once a day, supplemented with vitamin dried flake (Priyanka Enterprises Pvt Ltd, Nellore, India) food once a day.

Embryos were collected from this stock by keeping the spawning glass trays covered with fine nylon net with an appropriate mesh size of eggs to fall through, in the evening before the collection was made. Plant imitations made of plastic, which serves as spawning substrate are fastened to the net. Embryos were collected in the dawn of next morning. Quality was checked under microscope to select the healthy embryos.

Embryo-larval toxicity treatment

Embryos of the same developmental stage (4hpf) were selected and were transferred to six petridishes (each containing 100 embryos) containing 200mL of embryonic medium (1-5mM NaCl, 0.17mM KCl, 0.33mM CaCl2, 0.33mM MgSO4). They were exposed to two concentrations of DM namely 100(2µl/100ml) and 200µg/L (4µl/100ml) and other served as solvent controls (only solvent added) and controls (without solvent and toxicant) separately for each concentration. These were maintained at an ambient temperature of about 27±1^{o}C in a static condition and fresh media were renewed daily along with DM; and stirred for uniform distribution. Embryos were examined for any mortality after 24h and 48h; dead embryos/larvae were removed at frequent intervals. Three sets of exposures were carried for each group. Samples were collected after 12hpf, 24hpf and 48hpf for RT-PCR analysis.

Western blot Analysis

The protocol was prepared by the method of Sambrook et al.[18] 100µg of protein samples was resolved in 7% SDS-PAGE gels along with the protein molecular weight standards, and then transferred onto Nitrocellulose membrane. Once the transfer process was finished, the membrane was stained with ponceau reagent to check the transfer of protein onto the PVDF membrane. The ponceau was removed by raising the membrane with 1X TBS-T buffer (12g Tris, 9gm Nacl and 500µl Tween 20). The membranes were blocked with 5% w/v blocking buffer of prevent any non specific binding of anti bodies. Blocking was continued for 1 hour at room temperature with constant shaking. Then the membrane was washed three times for 15 minutes in large volumes of 1X TBS-T buffer. Required quantity of primary antibodies specific for *flh*, *ntl*, *doc* and *syu* genes were added to 10ml of buffer (1X Tris-buffered saline with 0.05% Tween 20 with 5% milk). The blocked membrane is incubated in this buffer with gentle shaking at 4^{o}C for 8-12hr. after primary antibody incubation, the membrane was incubated for 1hr at room temperature with peroxidase conjugated secondary antibodies. Then the membrane was washed three times with 1X TBS-T buffer and protein signals were detected by using an ECL western blotting kit.

Reverse transcription - Polymerase chain reaction (RT-PCR)

Isolation of RNA by TRIzol Method

Total RNA was isolated from Zebrafish embryos/Larvae of 12hpf, 24hpf and 48hpf developmental stages of both control and treated groups using TRIzol reagent. The protocol was followed as per manufacturer's instructions. RNA quantities were assessed using the Nano Drop ND-2000 spectrophotometer (Thermo Scientifics) by measuring at A260/A280nm.

Quality and Quantity Analysis of RNA

Quality analysis of RNA was done by 1.2% Agarose gel electrophoresis with 1XTBE buffer (Tris, EDTA). Equal volumes of RNA mixed with RNA tracking dye and was loaded along with RNA ladder on to the gel slots and electrophoresed for 60minutes at 100V. After complete run 2 bright bands were visible indicating 18S and 23S RNA. The isolated RNA quantity was measured at 260/280 nm using the Nano Drop Spectrophotometer. RNA was collected and stored at -80ºC.

Synthesis of cDNA

Total RNA obtained was used for synthesizing DNA by first strand cDNA synthesis kit (Thermo Scientifics) according to the manufacturer's instruction. In brief 1µg

of RNA sample was mixed with 1µl of oligo dT, 0.25µl of RNase inhibitor (10U/µl) and 10µl of DEPC water. The reaction mixture was kept at 65°C for 15 min and immediately chilled on ice. Then 4µl of 5x reaction buffer, 2µl of dNTP's (10mM), 1µl of Reverse Transcriptase enzyme (200U/µl) was added, mixed gently, centrifuged briefly and incubated at 37°C for 60 min. After incubation was completed, the reaction was terminated by heating at 70°C for 10min and immediately chilled on ice. Quantity and purity of cDNA were measured at A260/A280nm using Nano Drop ND-2000 spectrophotometer (Thermo Scientifics). The obtained cDNA was confirmed by Agarose Gel electrophoresis and visualized using UV-transilluminator.

Primer Designing

In order to monitor PCR amplification with maximum specificity and efficiency, RT-PCR primers were designed using Primer3 software. Criteria considered are: Melting temperature Tm>25°C, GC content of 40-60%, Primer length 20-24 nucleotides and amplicon size of 100-200bp. Primer sequence for selected genes (Table 1).

STATISTICAL ANALYSIS

Standard deviation and level of significance was calculated for data obtained from three replicates. Two-way ANOVA was carried out by using Graph Pad Prism software version 6.0.

RESULTS

At 12hpf "Fig. 1" and "Fig. 1A" and 24hpf "Fig. 2 and "Fig. 2A" stage all four genes namely *ntl*, *flh*, *doc* and *syu* showed a significant (P<0.05) increase over control in 200µg/L treated group. *syu* gene showed more expression followed by *ntl*, *flh and doc* at 12hpf "Fig. 1" and "Fig. 1A" stage. At 24hpf "Fig. 2 and 2A" stage *syu* gene showed more expression followed by *flh*, *ntl* and *doc*. At 48hpf "Fig. 3" and "Fig. 3A" all four genes showed significant increase of expression compared to control in both treated groups (100 and 200µg/L). *flh* gene showed more expression followed by *ntl*, *doc* and *syu*.

We have analysed the protein *ntl*, *flh*, *doc* and *syu* by western blot at three developmental stages (12hpf, 24hpf and 48hpf) exposed to two different concentrations of DM i.e., 100 and 200µg/L. Protein content at 12hpf, the first developmental stage and 48hpf, the last developmental stage of our study exposed to 100 and 200µg/L are shown in "Fig. 4" and "Fig. 4A", "Fig. 6" and "Fig. 6A". Western blot analysis of *ntl*, *flh*, *doc* and *syu* have shown a significant (P<0.05) increase in DM treated embryos/larvae at all developmental stages (12hpf, 24hpf and 48hpf) over control in 200µg/L group. At both 12hpf and 48hpf developmental stages no significant increase of Protein exprsession in DM treated embryos/larvae was noticed in 100µ/L group. At 24hpf "Fig. 5" and "Fig. 5A" stage DM exposed embryos/larvae showed significant (P<0.05) increase of Protein exprsession in 100µg/L group.

Comparison between these four *ntl*, *flh*, *doc* and *syu* at 12hpf "Figure 4" and "Fig. 4A" stage *doc* protein content was high followed by *syu*, *ntl* and *flh* in both treated groups (100 and 200µg/L). *flh* protein content was more at 24hpf, "Fig. 5 and "Fig. 5A" stage followed by *doc*, *ntl* and *syu* in both concentrations. At 48hpf, "Fig. 6 and "Fig. 6A" stage *ntl* protein content was high followed by *syu*, *flh* and *doc* in both exposure groups. All four showed more percent increase in both DM treated groups over control.

Table I: Primers used for Reverse transcriptase – PCR analysis of the following genes.

Gene		Primer Sequence	Length	Temperature
ntl	FP	5'- AAGGAGGTTGCTGATCGTGG -3'	20	54.5^{O}C
	RP	5'- CTCTGCACTCCAAGTCCCAT-3'	20	
flh	FP	5'- CGAAAGCAGCAGTTCATTCTC-3'	21	25.5^{O}C
	RP	5'- CAGATGCCAACAGAAAGCGT-3'	20	
syu	FP	5'- GATACGAGGGCAAGATAACG-3'	20	52.6^{O}C
	RP	5'- GTGACCGTCCTCATCCCA -3'	18	
doc	FP	5'- TCAGTGTGTGACGAGGACAA-3'	20	56.8^{O}C
	RP	5'- TGCTGAAGTTCTTGGTCTGG -3'	20	

ILLUSTRATIONS AND FIGURES

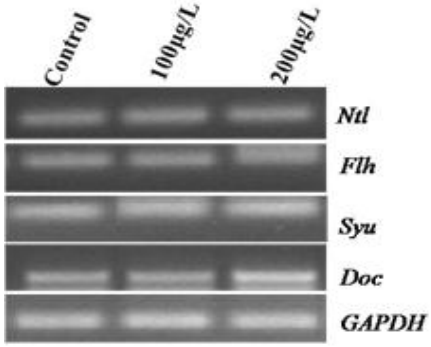


Fig. 1

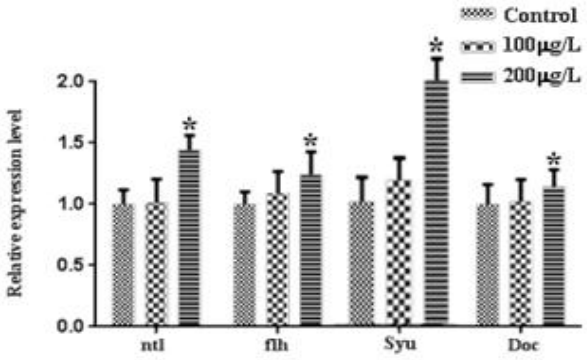


Fig. 1A

Fig: 1. Embryo/larval samples of zebrafish after exposure to 100 and 200µg/L of DM were analyzed by RT-PCR for expression of *ntl*, *flh*, *syu* and *doc* at 12hpf.

Fig: 1A. Graphical expression of four different genes relative to GAPDH levels in 12hpf embryos of Zebrafish (*Danio rerio*) exposed to 100 and 200µg/L of DM. Values are mean of embryos/larval samples (n = 100) ± SD. Increment was statistically significant (P<0.05) over control when denoted with asterik (*).

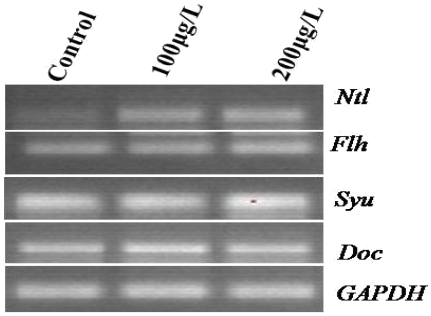


Fig. 2

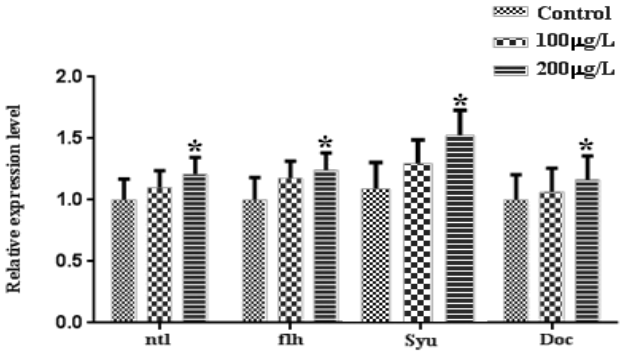


Fig. 2A

Fig: 2. Embryo/larval samples of zebrafish after exposure to 100 and 200µg/L of DM were analyzed by RT-PCR for expression of *ntl*, *flh*, *syu* and *doc* at 24hpf

Fig: 2A. Graphical expression of four different genes relative to GAPDH levels in 24hpf embryos of Zebrafish (*Danio rerio*) exposed to 100 and 200µg/L of DM. Values are mean of embryos/larval samples (n = 100) ± SD. Increment was statistically significant (P<0.05) over control when denoted with asterik (*).

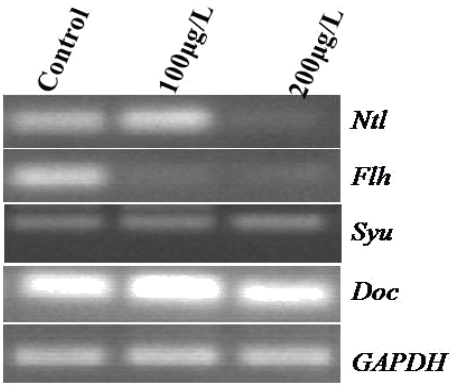


Fig. 3

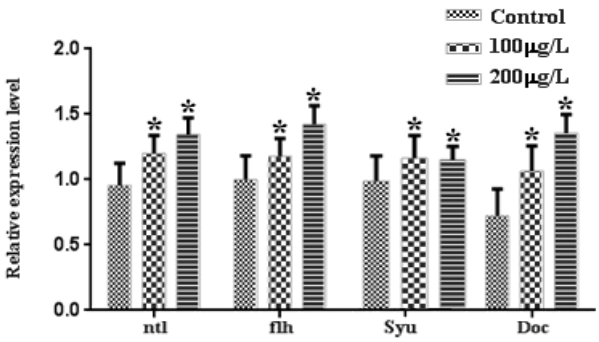
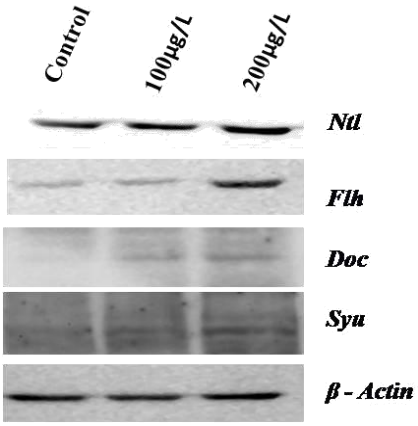


Fig. 3A

Fig: 3. Embryo/larval samples of zebrafish after exposure to 100 and 200µg/L of DM were analyzed by RT-PCR for expression of *ntl*, *flh*, *syu* and *doc* at 48hpf.

Fig: 3A. Graphical expression of four different genes relative to GAPDH levels in 48hpf embryos of Zebrafish (*Danio rerio*) exposed to 100 and 200µg/L of DM. Values are mean of embryos/larval samples (n = 100) ± SD. Increment was statistically significant (P<0.05) over control when denoted with asterik (*).



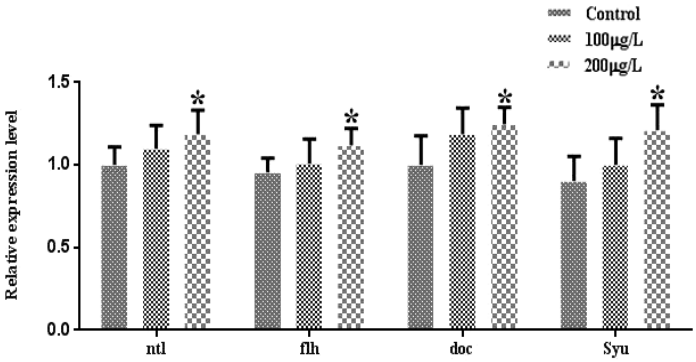
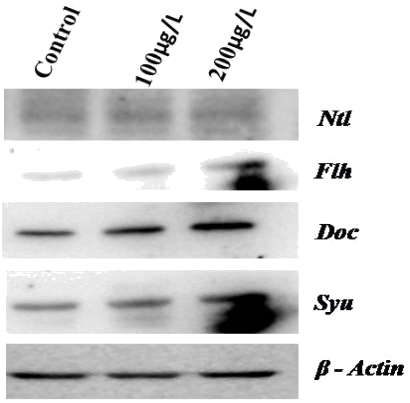


Fig. 4 **Fig. 4A**

Fig: 4. Embryo/larval samples of zebrafish after exposure to 100 and 200µg/L of DM were analyzed by Western blot for expression of *ntl, flh, doc and syu* at 12hpf.

Fig: 4A. Graphical expression of four different genes relative to β-Actin levels in 12hpf embryos of Zebrafish (*Danio rerio*) exposed to 100 and 200µg/L of DM. Values are mean of embryos/larval samples (n = 100) ± SD. Increment was statistically significant (P<0.05) over control when denoted with asterik (*).



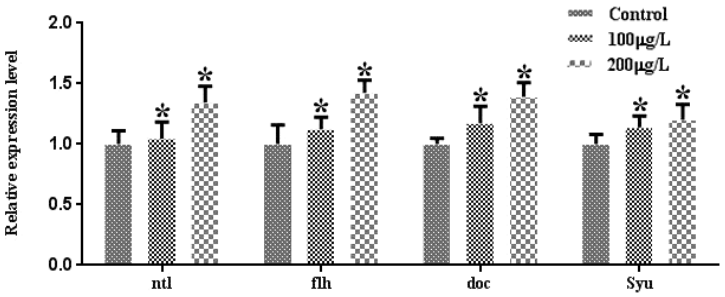
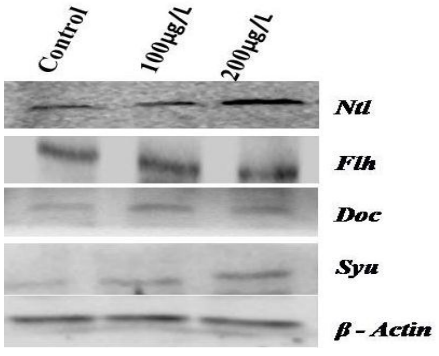


Fig. 5 **Fig. 5A**

Fig: 5. Embryo/larval samples of zebrafish after exposure to 100 and 200µg/L of DM were analyzed by Western blot for expression of *ntl, flh, doc and syu* at 24hpf.

Fig: 5A. Graphical expression of four different genes relative to β-Actin levels in 24hpf embryos of Zebrafish (*Danio rerio*) exposed to 100 and 200µg/L of DM. Values are mean of embryos/larval samples (n = 100) ± SD. Increment was statistically significant (P<0.05) over control when denoted with asterik (*).



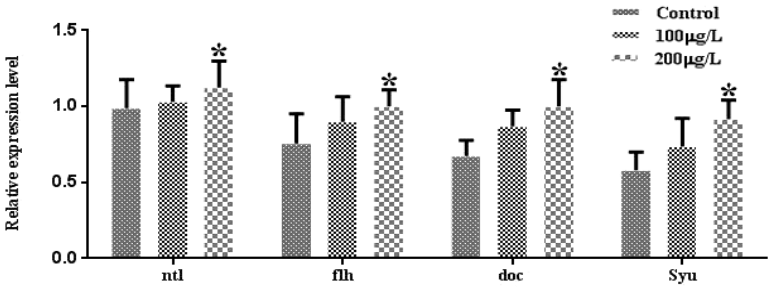


Fig. 6 **Fig. 6A**

Fig: 6. Embryo/larval samples of zebrafish after exposure to 100 and 200µg/L of DM were analyzed by Western blot for expression of *ntl, flh, doc and syu* at 48hpf.

Fig: 6A. Graphical expression of four different genes relative to β-Actin levels in 48hpf embryos of Zebrafish (*Danio rerio*) exposed to 100 and 200µg/L of DM. Values are mean of embryos/larval samples (n = 100) ± SD. Increment was statistically significant (P<0.05) over control when denoted with asterik (*).

DISCUSSION

Expression pattern of four genes namely *ntl, flh, doc* and *syu* were examined after exposing 4hpf embryos to DM for two days. All four genes are known to be involved in the formation of notochord during development of zebrafish.[19],[20],[21],[22] In zebrafish, notochord formation starts from 10.5hpf stage and is completed by the end of the day. For this reason this window of exposure was selected. Also studies from our lab have shown that DM (Decis) has caused dorsal curvature of body.[23] Research studies on relationship between phenotypic abnormalities and pattern of expression of related genes has been less focused. The current study deals with the phenotypic abnormalities and their respective developmental genes and proteins expression in DM exposed zebrafish embryos/larvae. Our study shows expression pattern of all four genes at all the three stages of development i.e., 12hpf, 24hpf and 48hpf after exposure to both (100 and 200µg/L) concentrations of DM was enhanced.

Notochord is a defined structure of all chordates including zebrafish. This was affected to a larger extent during early development. The notochord provides axial support to the growing embryo, which is essential for subsequent vertebral column formation; secretes signaling molecules required for neural patterning, muscle differentiation, cardiac formation[24] and also plays a crucial role in somite patterning.[25] Due to mutations in *you* and *yot* genes, the adaxial cells and the muscle pioneers are reduced or absent. Adaxial cells form next to the notochord, eventually giving rise to slow muscle.[26], [27] Improper formation of muscles could be the reason for abnormal development of tail. This prominent organ is easily visualized, consisting of a series of notochord vacuolar cells that exert turgor pressure on an extracellular matrix sheath.[28] Therefore from the observations it can be said these are different end points for DM action.

According to Fredericus et al.[29], mutations in *ntl, flh* and *doc* genes have resulted in embryos lacking muscle pioneer cells, which forms following induction of adaxial cells through signaling from the notochord and a horizontal myoseptum, which separates the somites into dorsal and a ventral part. Mutations in these genes were also known to affect the early specification of the notochord primordium.[29] They have also suggested that function for *doc* in the maintenance of *ntl* expression. The zebrafish shh orthologue is named *syu* (Sonic-you).[30] In comparision with mouse embryos, the *syu* phenotype is very mild in the neural tube of zebrafish embryos as the medial floor plate and motor neurons appear to form normally; lateral floor plate cells, however, are missing in *syu* mutants.

In zebrafish *ntl* and *flh* genes have been identified for notochord development. In our present study, we observed that DM induced expression of all four genes *ntl, flh, doc* and *syu*. Talbot et al.[22], showed mutations in the *flh* gene was known to be the cause for lack of notochord along the entire length of the embryo, with fused somites in the trunk. These somites defects could be due to an impairment of the specification of the adaxial cells during early stages of development as suggested by Odenthal et al.[20] They have also shown the expression of this gene in the notochord and floor plate of early zebrafish embryos. Halpern et al.[31], carried out expression studies and suggested that cells lacking *flh* function can only differentiate into muscle but not notochord. *ntl* along with its role in notochord development it also involved in tail formation.[19],[32]

In *ntl* (no tail) mutant embryos which are defective in tail formation and proper morphogenesis of the notochord. Thus it can be said that, the *ntl* gene product, a transcription factor, was required for the expression of connexin43.4 in both the notochord and tail bud during morphogenesis.[33] These were also involved in cell interactions associated with morphogenetic expression of connexin and as such can alter the permeability of cell movements and cell adhesion.[34]

The *flh* protein, a homeodomain protein, is thought to act in cells upstream of the *ntl* gene product during notochord differentiation. *flh* mutant embryos showed a reduced expression of the *ntl* gene in the axial hypo blast.[22] Toward end of gastrulation in wild-type embryos, the axial hypo- blast begins morphogenesis into the notochord and creates a visible boundary between the notochordal precursor cells and the paraxial hypoblast. This boundary is not observed in *ntl* mutant embryos even though notochordal precursor cells are still present.[19]

The genes *pbx1, foxA3*[35], [36], [37], *mnx1, has2, anXa5b, hprt1l* and *elovl1a*[38], [39], [40], [37], [41] are essential for the development of swim bladder. In a study by Strungaru et al.[42] all these genes essential for development of swim bladder and were all significantly down regulated in response to DM treatment, suggesting that DM might directly and specifically inhibited swim bladder development. Moreover, *pbx1a* is required for anlage development in swim bladder from 28hpf[36] and *foxA3* is typically expressed in the swim bladder bud and other gut-derived organs.[43] Hence, the significantly reduced transcription levels of *pbx1a* and *foxA3* in the present study suggested the impacts of DM on swim bladder inflation might be tracked as early as the epithelial bud initiation stage. The *mnx1* and *has2* are marker genes for the development of epithelium and mesenchyme, respectively. The *anxa5, hprt1l* and *elovl1a* can be used as marker genes of mesothelial development.[40], [37]

CONCLUSION

We have exposed 12hpf, 24hpf and 48hpf zebrafish embryos to two concentrations (100 and 200µg/L) of DM to analyzed genes and proteins which are responsible for notochord and somite formation during development. Our results suggest that DM interferes with the expression of four genes in early development of

zebrafish. In our present study, we observed that DM induced in expression of all genes. Up regulation of genes along with its protein as evidenced by the western blot. This shows that DM stepped up both the process namely transcription and translation.

ACKNOWLEDGEMENTS

1. One of the authors K. Reshma Shabnam is grateful to Department of science and Technology, New Delhi for the financial support through INSPIRE Fellowship, Inspire Fellow number (IF 140051).

REFERENCES

1. Luo Y & Zhang M, Environmental Modeling and Exposure Assessment of Sediment-Associated Pyrethroids in an Agricultural Watershed, 2011; 6.
2. Agency for Toxic Substances and Disease Registry (ATSDR), Atlanta, GA. (2003). http://www.atsdr.cdc.gov/gsl/getsite.
3. Ding Y, White CA, Muralidhara S, Bruckner JV, Bartlett MG, Determination of deltamethrin and its metabolite 3-phenoxybenzoic acid in male rat plasma by high-performance liquid chromatography. Journal of Chromatography B, 2004; 810(2): 221-227.
4. ANVISA, Consulta Pública n^{o} 64, de 11 de Julho de, 2007.
5. U.S. EPA. EPA's Report on the Environment (ROE) (2008 Final Report). U.S. Environmental Protection Agency, Washington, D.C., EPA/600/R-07/045F (NTIS PB2008-112484), 2008.
6. DeMicco A, Cooper KR, Richardson JR, & White LA, Developmental Neurotoxicity of Pyrethroid Insecticides in Zebrafish Embryos. Toxicol Sci, 2010; 113(1): 177-186.
7. Berkowitz GS, Obel J, Deych E, Lapinski R, Godbold J, Liu Z, Landrigan JP & Wolff SM, Exposure to indoor pesticides during pregnancy in a multiethnic, urban cohort. Environ Health Perspect, 2003; 111(1): 79-84.
8. Whyatt RM, Camann DE, Kinney PL, Reyes A, Ramirez J, Dietrich J, Diaz D, Holmes D & Perera FP, Residential pesticide use during pregnancy among a cohort of urban minority women. Environmental Health Perspectives, 2002; 110(5): 507-514.
9. Lu C, Barr D, Pearson M, Bartell S & Bravo R, A longitudinal approach of assessing urban and suburban children's exposure to pyrethroid pesticides. Environ Health Perspect, 2006a; 114(9): 1419-1423.
10. Lu C, Barr DB, Pearson MA, Walker LA & Roberto Bravo, The attribution of urban and suburban children's exposure to synthetic pyrethroid insecticides: a longitudinal assessment. J Expo Sci Environ Epidemiol, 2009; 19(1): 69-78.
11. Morgan MK, Sheldon LS, Croghan CW, Jones PA, Chuang JC & Wilson NK, An observational study of 127 preschool children at their homes and day care centers in Ohio: environmental pathways to cis- and trans-permethrin exposure. Environmental Research, 2007; 104(2): 266-274.
12. He F, Wang S, Liu L, Chen S, Zhang Z & Sun J, Clinical manifestations and diagnosis of acute pyrethroid poisoning. Arch. Toxicol, 1989; 63(1): 54-58.
13. Reshma Shabnam K & Harold Philip G. Developmental toxicity of Deltamethrin and 3-Phenoxybenzoic acid in embryo-larval stages of Zebrafish (Danio rerio). Toxicology Mechanisms and Method, 2018; 28(6): 415-422.
14. Shamshad Begam S, Suvarchala G & Philip GH, Protein profile in tissues of zebrafish exposed to deltamethrin. J. Bio. Innov, 2016; 5(6): 914-922.
15. Pullanna K & Philip GH, Reproductive performance of zebrafish (Danio rerio) exposed to deltamethrin: Fecundity, histologogical and hormonal end points. Journal of Experimental and Applied Science, 2014; 1(2): 253-264.
16. Reshma Shabnam Kuder & Gundala Harold Philip**,** Antioxidant enzymatic activities and lipid peroxidation in liver and ovary of zebrafish (Danio rerio) exposed to deltamethrin. Chemistry and Ecology, 2017; 33(8): 739-749.
17. Hill AJ, Teraoka H, Heidemann W & Peterson RE, Zebrafish as model vertebrate for investigating chemical toxicity. Toxicological Sciences, 2005; 86(1): 6-19.
18. Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989; 3: 12.
19. Halpern ME, Ho RK, Walker C & Kimmel CB, Induction of muscle pioneers and floor plate is distinguished by the zebrafish no tail mutation. Cell, 1993; 75(1): 99-111.
20. Odenthal J, Haffter P, Vogelsang E & Brand M, Mutations affecting the formation of the notochord in the zebrafish, Danio rerio. Development, 1996; 123: 103-115.
21. Stemple DL, Solnica-Krezel L, Zwartkruis F, Neuhauss SC, Schier AF, Malicki J, Stainier DY, Abdelilah S, Rangini Z, Mountcastle-Shah E & Driever W, Mutations affecting development of the notochord in zebrafish. Development, 1996; 123: 117-128.
22. Talbot WS, Trevarrow B, Marnie E. Halpern ME, Anna E. Melby AE, Gist Farr, Postlethwait JH, Trevor Jowett, Kimmel CB & David Kimelman, A homeobox gene essential for zebrafish notochord development. Nature, 1995; 378(6553): 150-157.
23. Nalini, Effect of synthetic pyrethroid, Deltamethrin on hatching and development in Zebrafish, Danio rerio, (2012) Thesis.
24. Stemple DL, Structure and function of the notochord: an essential organ for chordate development. Development, 2005; 132(11): 2503-2512.

25. Bumcrot DA & McMahon AP, Somite differentiation. Sonic signals somites. Curr Biol**,** 1995; 5(6): 612-614.
26. Van Raamsdonk W, Pool CW & te Kronnie G, Differentiation of muscle fibre types in the teleost Brachy Danio rerio. Anat. Embryol, 1978; 153(2): 137-155.
27. Devoto SH, Melancon E, Eisen JS & Westerfield M Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation. Development, 1996; 122(11): 3371-3380.
28. Scott A & Stemple DL, Zebrafish notochordal basement membrane: signaling and structure. Curr Top Dev Biol, 2005; 65: 229-253.
29. Fredericus JM, Eden V & Granto M. 1996. Mutations affecting somite formation and patterning in the zebrafish, Danio rerio. Development, 1996; 123: 153-64.
30. Schauerte HE, van Eeden FJ, Fricke C, Odenthal J, Strähle U & Haffter P, Sonic hedgehog is not required for the induction of medial floor plate cells in the zebrafish**.** Development, 1998; 125(15): 2983-2993.
31. Halpern ME, Thisse C, Ho RK & Thisse B, Cell-autonomous shift from axial to paraxial mesodermal development in zebrafish floating head mutants. Development, 1995; 121(12): 4257-4264.
32. Schulte-Merker S, van Eeden FJ & Halpern ME, No tail (ntl) is the zebrafish homologue of the mouse T (Brachyury) gene. Development, 1994; 120(4): 1009-15.
33. Essner JJ, Laing JG & Beyer EC, Expression of Zebrafish connexin43.4 in the Notochord and Tail Bud of Wild-Type and Mutant no tail Embryos. Developmental Biology, 1996; 177(2): 449-462.
34. Wilson V, Manson L, Skarnes WC & Beddington RSP, The T gene is necessary for normal mesodermal morpho-genetic movements during gastrulation. Development, 1995; 121(3): 877-886.
35. Muller II, Knapik EW & Hatzopoulos AK, Expression of the protein related to Dan and Cerberus gene-prdc- During eye, pharyngeal arch, somite, and swim bladder development in zebrafish. Dev Dyn, 2006; 235(10): 2881-2888.
36. Teoh PH, Shu-Chien AC & Chan WK, Pbx1 is essential for growth of zebrafish swim bladder. Dev. Dyn, 2010; 239(3): 865-874.
37. Yin A, Korzh S, Winata CL, Korzh V & Gong Z, 2011. Wnt signaling is required for early development of zebrafish swimbladder. PLoS One, 2011; 6(3): 18431.
38. Mccune AR & Carlson RL, Twenty ways to lose your bladder: common natural mutants in zebrafish and widespread convergence of swim bladder loss among teleost fishes. Evol. Dev, 2004; 6(4): 246-259.
39. Wendik B, Maier E & Meyer D, 2004. Zebrafish mnx genes in endocrine and exocrine pancreas formation. Dev. Biol, 2004; 268(2): 372-83.
40. Winata CL, Korzh S, Kondrychyn I, Zheng W, Korzh V & Gong Z, Development of zebrafish swimbladder: the requirement of Hedgehog signaling in specification and organization of the three tissue layers. Dev. Biol, 2009; 331(2): 222-236.
41. Bhandari S, Lee JN, Kim YI, Nam IK, Kim SJ, Kim SJ, Kwak S, Oh GS, Kim H.J, Yoo HJ, So HS, Choe SK & Park R, The fatty acid chain elongase, Elovl1, is required for kidney and swim bladder development during zebrafish embryogenesis. Organogenesis, 2016; 12(2): 78-93.
42. Strungaru SA, Plavan G, Ciobica A, Nicoara M, Robea MA, Solcan C & Petrovici A, Toxicity and chronic effects of deltamethrin exposure on zebrafish (Danio rerio) as a reference model for freshwater fish community. Ecotoxicol. Environ. Saf, 2019; 171: 854-862.
43. Ober EA, Field HA & Stainier DY, From endoderm formation to liver and pancreas development in zebrafish. Mech. Dev, 2003; 120(1): 5-18.